Hypoxia-Inducible Factor Signaling Provides Protection in *Clostridium difficile*-Induced Intestinal Injury

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BACKGROUND & AIMS: *Clostridium difficile* is the leading cause of nosocomial infectious diarrhea. Antibiotic resistance and increased virulence of strains have increased the number of *C difficile*-related deaths worldwide. The innate host response mechanisms to *C difficile* are not resolved; we propose that hypoxia-inducible factor (HIF-1) has an innate, protective role in *C difficile* colitis. We studied the impact of *C difficile* toxins on the regulation of HIF-1 and evaluated the role of HIF-1α in *C difficile*-mediated injury/inflammation. **METHODS:** We assessed HIF-1α mRNA and protein levels and DNA binding in human mucosal biopsy samples and Caco-2 cells following exposure to *C difficile* toxins. We used the mouse ileal loop model of *C difficile* toxin-induced intestinal injury. Mice with targeted deletion of HIF-1α in the intestinal epithelium were used to assess the effects of HIF-1α signaling in response to *C difficile* toxin. **RESULTS:** Mucosal biopsy specimens and Caco-2 cells exposed to *C difficile* toxin had a significant increase in HIF-1α transcription and protein levels. Toxin-induced DNA binding was also observed in Caco-2 cells. Toxin-induced HIF-1α accumulation was attenuated by nitric oxide synthase inhibitors. In vivo deletion of intestinal epithelial HIF-1α resulted in more severe, toxin-induced intestinal injury and inflammation. In contrast, stabilization of HIF-1α with dimethyloxallyl glycine attenuated toxin-induced injury and inflammation. This was associated with induction of HIF-1-regulated protective factors (such as vascular endothelial growth factor-α, CD73, and intestinal trefoil factor) and down-regulation of proinflammatory molecules such as tumor necrosis factor and Cxcl1. **CONCLUSIONS: HIF-1α protects the intestinal mucosa from *C difficile* toxins. The innate protective actions of HIF-1α in response to *C difficile* toxins be developed as therapeutics for *C difficile*-associated disease.**

Keywords: Vascular Endothelial Growth Factor; Tumor Necrosis Factor; ITF/TFF3; Nitric Oxide Synthase.

Clostridium difficile is a spore-forming, toxin-producing (toxin A [TcdA], 308 kilodaltons; toxin B [TcdB], 270 kilodaltons) bacteria[1] that is responsible for a concerning epidemic. The recent emergence of NAP-1/027 strains, which are more virulent and resistant to antibiotics, has magnified the impact of the current *C difficile* crisis. Alarmingly, *C difficile*-related deaths in the United States have risen by 35% per year since 1999[2] so that *C difficile* now causes twice the number of deaths as human immunodeficiency virus. Even with the best medical therapy, patients can rapidly progress to toxic megacolon, sepsis, and death.

C difficile toxins rapidly induce intestinal injury and inflammation through disruption of the intestinal epithelial barrier and induction of proinflammatory mediators.[3] Both TcdA and TcdB are glucosyltransferases that irreversibly inactivate the Ras superfamily of small guanosine triphosphatases.[1] TcdA-induced changes in the actin cytoskeleton disrupts tight junctions, induces cell rounding, and detachment.[4–6] Following detachment, intestinal epithelial cells can be driven to produce interleukin (IL)-8 and up-regulate intercellular adhesion molecule-1[7] leading to the neutrophil chemoattraction, adhesion, and subsequent mucosal inflammation.[8] The disruption of the mucosal barrier allows toxins to activate the immune cells within the lamina propria and to trigger the production of IL-8, IL-1β, tumor necrosis factor (TNF), and nitric oxide (NO).[7–10] These events can lead to further immune cell infiltrate and the tissue

Abbreviations used in this paper: CDAD, *C difficile*-associated disease; DMOG, dimethyloxaloylglycine; HIF-1, hypoxia-inducible factor-1; HIF-1$\alpha^{(Ep-/-)}$, targeted epithelial HIF-1$\alpha^{-/-}$ knockout; HRE, hypoxia-responsive element; IL, interleukin; ITF/TFF3, intestinal trefoil factor; L-NAME, L-Nitro-Arginine Methyl Ester; LP, lamina propria; mRNA, messenger RNA; NF-κB, nuclear factor-κB; PCR, polymerase chain reaction; PHD, prolyl hydroxylases; PMN, polymorphonuclear cells; TER, transepithelial resistance; TcdA, toxin A; TcdB, toxin B.

0016-5085/$36.00
doi:10.1053/j.gastro.2010.03.045

damage associated with *C difficile*-associated disease (CDAD).

The hypoxia-inducible factor-1 (HIF-1) transcription factor complex responds rapidly to cellular stress, injury, and inflammation and can protect the intestinal mucosa.[11–13] HIF-1, composed of an oxygen-labile α-subunit and a nuclear-localized β-subunit, is a master transcriptional factor and prototypical regulator of oxygen-dependent gene transcription. The HIF-1α subunit is ubiquitously and constitutively expressed, but, in normoxic conditions, the protein is subject to hydroxylation by prolyl hydroxylases (PHD). This event triggers the binding of von Hippel Lindau protein, which targets HIF-1α to the proteasome for degradation.[14] Upon stabilization during hypoxia, in the absence of hydroxylation, the HIF-1α-subunit translocates to the nucleus where it associates with its HIF-1β subunit and initiates transcriptional events by binding to hypoxia responsive elements (HREs) present in promoter or enhancer regions of various genes. Thus, events that prevent the degradation of the HIF-1α-subunit trigger potent HIF-1-mediated transcription events.[14,15]

In addition to promoting the adaptive responses to hypoxia, HIF-1 signaling has regulatory actions in a wide variety of cellular processes involved in immune[13] and intestinal barrier function.[16–19] HIF-1 can increase the transcription of genes (eg, vascular endothelial growth factor [VEGF], endothelial nitric oxide synthase [eNOS], inducible nitric oxide synthase [iNOS], and intestinal trefoil factor [ITF]) that are involved in wound repair, inflammation, cellular permeability, and apoptosis.

The transcriptional activation of HIF-1 can also be induced in normoxia by a number of cytokines, growth factors, and other circulating molecules (eg, nitric oxide [NO]) through stabilization of the HIF-1α subunit.[13] Indeed, HIF-1 can attenuate several aspects of the inflammation/injury cascade and promote the expression of a suite of barrier-protective genes including intestinal trefoil factor (TFF3/ITF), mucin family members (MUC-1), and ecto-5'-nucleotidase (CD73).[18] These observations led us to hypothesize that HIF-1α stabilization and downstream HIF-1 signaling events play critical innate protective roles in *C difficile*-induced intestinal inflammation and injury.

To test this hypothesis, we assessed whether *C difficile* toxins could elevate HIF-1α messenger RNA (mRNA) and protein levels and influence HIF-1 DNA-binding activity in a human colonic epithelial cell line. We also assessed HIF-1α expression in patients with CDAD and in healthy mucosal biopsy specimens exposed to *C difficile* toxin. Finally, we employed the mouse ileal loop model to examine the effect of *C difficile* toxins on intestinal inflammation in animals with knock-down of HIF-1α targeted to the intestinal epithelium or animals treated with dimethyloxaloylglycine (DMOG), a PHD inhibitor, to inhibit the degradation of HIF-1α in vivo.[16]

Materials and Methods

Human Tissue Sampling and HIF-1α Immunohistochemistry

Colonic tissue sections obtained from patients undergoing colonic resection for CDAD were probed with HIF-1α antibody (NB100-105) using standard immunohistochemical methodology and appropriate controls. Slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich, Oakville, Ontario). All studies were approved by the Conjoint Health Research Ethics Board of the University of Calgary/Calgary Health Region.

C difficile *Toxin Isolation and Purification*

C *difficile* strain NAP-1/027 ($TcdA^+/TcdB^+$; obtained from the Calgary Health Region) was grown in brain-heart infusion media under sterile anaerobic conditions. Cultures were harvested at day 5 postinoculation by centrifugation (10,000*g*, 60 minutes). TcdA and TcdB were purified from concentrated and filtered culture supernatants by anion-exchange chromatography as described previously[20] (see Supplementary Materials and Methods for complete method).

Assessment of mRNA Expression: RNA Isolation, Semiquantitative Reverse-Transcription Polymerase Chain Reaction, and Real-time Reverse-Transcription Polymerase Chain Reaction Analysis

Caco-2 or tissue biopsy specimens were collected in Trizol and RNA isolated according to manufacturer's protocol (Invitrogen, Carlsbad, CA). The total RNA was reverse transcribed with the RT[2] First-strand Kit (SABiosciences, Frederick, MD). The resulting complementary DNA was subjected to reverse-transcription polymerase chain reaction (RT-PCR) with specific primers (see Supplementary Table 1). Quantitative real-time polymerase chain reaction (PCR) was performed on an ABI 7500 real-time PCR thermocycler to quantify changes in gene expression. For Caco-2 cells, human biopsy specimen HIF-1α specific real-time primers were purchased (SABiosciences). For mouse ileum samples, specific primers for glyceraldehyde-3-phosphate dehydogenase, VEGFa, ITF, CD73, tumor necrosis factor (TNF), and KC/CXCL1 were purchased (SABiosciences). Amplification plots were examined with ABI-7500 Sequence Detection Software to determine the threshold cycle. In all reactions, endogenous control was amplified, and the threshold cycle was determined.

Western Blot/Protein Analysis

Western immunoblots were performed on protein extracts from Caco-2 cells or tissue biopsy homogenates. We used mouse monoclonal anti-HIF-1α antibodies (NB100-105 and ab6489), rabbit polyclonal anti-β-actin antibody (Novus Biologicals, Littleton, CO), and secondary horseradish peroxidase-conjugated antibody to